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Kuwayama, H; Snippe, H; Derks, M; Roelofs, J; van Haastert, PJM

*Published in:*
Biochemical Journal

*DOI:*
10.1042/0264-6021:3530635



*Publication date:*
2001

# Identification and characterization of DdPDE3, a cGMP-selective phosphodiesterase from *Dictyostelium*

Hidekazu KUWAYAMA[1], Helena SNIPPE, Mari DERKS, Jeroen ROELOFS and Peter J. M. VAN HAASTERT[2]

*GBB, Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

In *Dictyostelium* cAMP and cGMP have important functions as first and second messengers in chemotaxis and development. Two cyclic-nucleotide phosphodiesterases (DdPDE 1 and 2) have been identified previously, an extracellular dual-specificity enzyme and an intracellular cAMP-specific enzyme (encoded by the *psdA* and *regA* genes respectively). Biochemical data suggest the presence of at least one cGMP-specific phosphodiesterase (PDE) that is activated by cGMP. Using bioinformatics we identified a partial sequence in the *Dictyostelium* expressed sequence tag database that shows a high degree of amino acid sequence identity with mammalian PDE catalytic domains (DdPDE3). The deduced amino acid sequence of a full-length DdPDE3 cDNA isolated in this study predicts a 60 kDa protein with a 300-residue C-terminal PDE catalytic domain, which is preceded by approx. 200 residues rich in asparagine and glutamine residues. Expression of the DdPDE3 catalytic domain in *Escherichia coli* shows that the enzyme has Michaelis–Menten kinetics and a higher affinity for cGMP ($K_m = 0.22\ \mu M$) than for cAMP ($K_m = 145\ \mu M$); cGMP does not stimulate enzyme activity. The enzyme requires bivalent cations for activity; $Mn^{2+}$ is preferred to $Mg^{2+}$, whereas $Ca^{2+}$ yields no activity. DdPDE3 is inhibited by 3-isobutyl-1-methylxanthine with an $IC_{50}$ of approx. 60 $\mu$M. Overexpression of the DdPDE3 catalytic domain in *Dictyostelium* confirms these kinetic properties without indications of its activation by cGMP. The properties of DdPDE3 resemble those of mammalian PDE9, which also shows the highest sequence similarity within the catalytic domains. DdPDE3 is the first cGMP-selective PDE identified in lower eukaryotes.

Key words: chemotaxis, cloning, guanylate cyclase, mutant *stm*F.

## INTRODUCTION

Eukaryotes are endowed with a large family of cyclic-nucleotide phosphodiesterase (*PDE*) genes [1–3]. The deduced amino acid sequences share a common element of approx. 300 amino acid residues that forms the catalytic domain. Some enzymes specifically hydrolyse cAMP (PDE4, PDE7 and PDE8) or cGMP (PDE5, PDE6 and PDE9), whereas other enzymes show dual specificity. Besides the putative catalytic domain, many enzymes possess additional domains that provide regulation by calmodulin (PDE1) or cGMP, which can be stimulatory (PDE2 or PDE5) or inhibitory (PDE3). The enzymic activities of PDEs might simply serve to degrade cAMP and cGMP. However, the complexity of the PDE superfamily provides the potential for additional regulatory circuits. For instance, the activation of guanylate cyclase and the accompanying increase in cGMP levels might indirectly alter the levels of cAMP, which will decrease when cAMP is hydrolysed mainly by a cGMP-stimulated cAMP-PDE and will increase when cAMP is hydrolysed by a dual-specificity enzyme that is subject to competitive inhibition by the increased cGMP levels.

In *Dictyostelium*, cAMP and cGMP have pronounced roles as first and second messengers [4,5]. *Dictyostelium* grows as single cells in the soil, feeding on bacteria. During this stage of the life cycle, cAMP and cGMP are probably not essential for growth. However, cyclic nucleotides are important when cells are starved. Depletion of the food source induces a developmental programme, leading to a multicellular stage. An extracellular cAMP signalling system is induced that is composed of the adenylate cyclase ACA, the G-protein-coupled cAMP surface receptor cAR1, and specific G-protein subunits. This signalling system produces extracellular cAMP that actually accumulates and disappears in an oscillatory manner. Degradation of cAMP by a PDE that is present in the extracellular medium and on the cell surface is essential to the generation of such cAMP pulses [6,7].

Extracellular cAMP induces chemotaxis: starving cells move towards the source of cAMP, thereby forming a slug-shaped multicellular structure. In this slug, cell differentiation towards pre-stalk and pre-spore cells takes place, finally leading to a fruiting body composed of a stalk tube supporting a droplet of spores. Intracellular cAMP is essential for pre-spore differentiation. It is synthesized by the G-protein-coupled adenylate cyclase ACA [8], and by a G-protein-independent enzyme ACB [9] encoded by the *acrA* gene [10]. Intracellular cAMP is degraded predominantly by a cAMP-specific enzyme encoded by the *regA* gene [11,12].

During cell aggregation, extracellular cAMP induces a rapid transient increase in cGMP levels [5]. This nucleotide is produced by a guanylate cyclase that is presumably directly activated by G-proteins. In cell lysates, cGMP is degraded predominantly by a cGMP-specific enzyme that is activated by cGMP [13–15]. The cGMP-stimulated cGMP-PDE activity from *Dictyostelium* is absent from *stm*F mutant NP368, which is characterized by prolonged chemotactic movement [14,16,17]. In this mutant, the cAMP-stimulated cGMP accumulation proceeds for a longer period, reaches higher concentrations and recovery of basal levels is retarded approx. 6-fold. Quantitative analysis of these data indicates that the cGMP-PDE that is absent from *stm*F accounts for approx. 80 % of the cGMP hydrolysis in wild-type cells and another unidentified enzyme for approx. 20 % [18].

Abbreviations used: 8Br-cGMP, 8-bromoguanosine 3′,5′-monophosphate; CIP, calf intestine alkaline phosphatase; EST, expressed sequence tag; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase.

[1] Present address: Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan.

[2] To whom correspondence should be addressed (e-mail p.j.m.van.haastert@chem.rug.nl).

To characterize further the role of cGMP in *Dictyostelium* chemotaxis, we have started to clone the genes that encode the enzymes synthesizing or degrading intracellular cGMP. The *Dictyostelium* sequencing project in Japan [19] provided a partial cDNA sequence encoding an amino acid sequence that exhibited a significant degree of identity with the catalytic domain of mammalian PDEs. We obtained a full-length sequence for this DdPDE3, and expressed the protein in *Escherichia coli* and *Dictyostelium*. DdPDE3 seems to degrade cGMP specifically but the enzyme is not activated by cGMP and is still present in mutant *stm*F. This suggests that DdPDE3 represents the residual cGMP-hydrolysing activity in mutant *stm*F.

## MATERIALS AND METHODS

### Materials

[2,8-$^3$H]cAMP (1.85 Bq/mmol) and [8-$^3$H]cGMP (0.55 Bq/mmol) were obtained from Amersham; cAMP, cGMP, 8-bromoguanosine 3′,5′-monophosphate (8Br-cGMP), calf intestine alkaline phosphatase (CIP) and IPTG (isopropyl $\beta$-D-thiogalactoside) were purchased from Boehringer Mannheim. 3-Isobutyl-1-methylxanthine (IBMX) was obtained from Calbiochem; pRSET came from Invitrogen.

### Strain and culture conditions

*Dictyostelium* AX3 ('wild-type'), PDE3 cells overexpressing DdPDE3, and A$^{15}$-Δ5′GCA/PDE3 cells overexpressing both DdGCA and DdPDE3, were grown in HG5 medium (HL5 medium with 10 g/litre glucose). When growing cells under selection, HG5 medium was supplemented with 10 $\mu$g/ml blasticidine S or 10 $\mu$g/ml geneticin (neomycin), depending on the selection marker present (blasticidine deaminase and neomycin phosphotransferase genes respectively). *Stm*F mutant NP368 and its isogenic parental strain XP55 were grown in association with *Klebsiella aerogenes*. Cells were starved for up to 5 h by being shaken in 10 mM phosphate buffer, pH 6.5, at a density of $10^7$ cells/ml for the indicated durations. For longer starvation periods, cells were transferred to non-nutrient agar plates [1.5 % (w/v) agar in 10 mM phosphate buffer, pH 6.5] and incubated at 22 °C. For DNA transfection by electroporation, cells were grown to exponential phase at a density of $(1–3) \times 10^6$ cells/ml. Approximately $10^7$ cells were mixed with 1–10 $\mu$g of DNA. After electroporation [20] and 5 h of recovery in a 10 cm dish, selection medium was added. Medium was changed after 24 h for the first time and thereafter approximately every 3 days.

### Cloning of DdPDE3

The expressed sequence tag (EST) clone SSB116 was identified as a potential PDE-encoding sequence in the Japanese cDNA project by using bioinformatics. The clone was kindly provided by Dr T. Morio [19] and sequenced completely. The insert was used as a probe to screen a $\lambda$ZAP cDNA library kindly provided by Dr R. A. Firtel. In a screen of $10^6$ clones, 21 positive clones were identified. Inserts from 14 clones were rescued as Bluescript plasmids, with insert sizes from 1.2 to 1.9 kb. LambdaZAP clone 116-6 had the largest insert and was sequenced completely.

### Northern and Southern analysis

RNA was isolated from $3 \times 10^7$ cells at the indicated developmental stage with the Qiagen Rneasy minikit by using the supplier's protocol for animal cells. After separation of the RNA samples on a formaldehyde/1 % (w/v) agarose gel, the samples were transferred to a Nytran filter (Schleicher & Schuell). Genomic DNA was purified with a minipreparation as described in [21]. Approximately 0.25 $\mu$g of genomic DNA was digested with the indicated restriction enzymes, separated on a 0.7 % agarose gel and transferred to a Nytran filter. Northern and Southern blots were preincubated for 2 h at 65 °C in hybridization solution [0.5 M sodium phosphate buffer (pH 7.0)/7 % (w/v) SDS/0.2 mg/ml BSA]. Hybridization was performed in the same prehybridization solution with the addition of a radioactive probe. Probes were labelled with $^{32}$P by the random primer method (High Prime; Boehringer Mannheim). Blots were incubated for 16 h, washed at high stringency with wash solution 1 [0.5M phosphate buffer/1 % (w/v) SDS (pH 7.0) at 65 °C] and wash solution 2 [0.1 M phosphate buffer/1 % (w/v) SDS (pH 7.0) at 65 °C] and exposed with a PhosphorImager.

### Expression of DdPDE3 catalytic domain in *E. coli*

A 0.9 kb region of cDNA 116-6 starting at bp 888 was amplified by PCR with the universal sequencing primer as the 3′ primer and the gene-specific 5′ primer 5′-ATCATGGATCCAAAA-TG**CGTGGTTATAATGATAATAATG**-3′; a *Bam*HI restriction site is underlined; the DdPDE3 sequence is indicated in bold. The PCR product was digested with *Bam*HI and *Kpn*I and subcloned in pUC21, yielding pUC21-PDE3. After sequencing, the *Bam*HI/*Kpn*I insert was ligated into the *Bam*HI and *Kpn*I sites of the bacterial expression plasmid pRSET-A, yielding pRSET-PDE3. The encoded protein lacks the first 201 residues of DdPDE3 consisting of the glutamine and asparagine repeats. Translation starts with the amino acid sequence MASMTGG-QQMGRGSKM (single-letter codes) derived from pRSET and the 5′ region of the PCR primer. It continues with the DdPDE3-derived amino acid sequence starting with R$^{205}$GYNDNN, which is approx. 35 amino acids before the start of the conserved catalytic domain.

Plasmid pRSET-PDE3 was used to transform *E. coli* BL21DE3. Bacteria were grown overnight at 37 °C in Luria–Bertani medium containing 25 $\mu$g/ml ampicillin and 50 $\mu$g/ml chloramphenicol. The culture was then diluted 1:40 in the same medium. After growth for 2 h at 30 °C, 25 $\mu$g/ml IPTG was added and growth was continued for 3 h at 30 °C. A 10 ml aliquot of the culture was centrifuged for 5 min at 3000 ***g***, the pellet was resuspended in 15 ml of ice-cold PDE buffer [20 mM Hepes/NaOH/1 mM $MgCl_2$ (pH 7.0)] and cells were lysed by sonication with four pulses of 3 s each. PDE activity was measured in the crude lysate; the activity was stable for several months when the lysate was stored at −80 °C.

### Expression of DdPDE3 catalytic domain in *Dictyostelium*

The *Bam*HI/*Xba*I insert of pUC21-PDE3 was ligated into the *Bgl*II/*Spe*I sites of the *Dictyostelium* expression plasmid HK12N, yielding HK12N-PDE3. The plasmid HK12N is a derivative of the extrachromosomal plasmid MB12N [22], with a small multiple cloning site in the original *Bgl*II site that allows the directional ligation of genes to be expressed. Translation from HK12N-PDE3 starts with the first ATG codon after the *Bam*HI site of the PCR primer; after translation the start methionine is followed by the DdPDE3-specific sequence beginning with the motif R$^{205}$GYNDNN.

HK12N-PDE3 was used to transform *Dictyostelium* wild-type strain AX3 and strain A$^{15}$-Δ5′GCA-neo, which overexpresses a guanylate cyclase under G418 selection (J. Roelofs, H. Snippe, R. G. Kleineidam and P. J. M. Van Haastert, unpublished work). Transformants were selected in HG5 medium in the presence of 10 $\mu$g/ml blasticidine. Cells were grown at 22 °C to a density of $3 \times 10^6$ cells/ml, washed in 10 mM phosphate buffer, pH 6.5, and

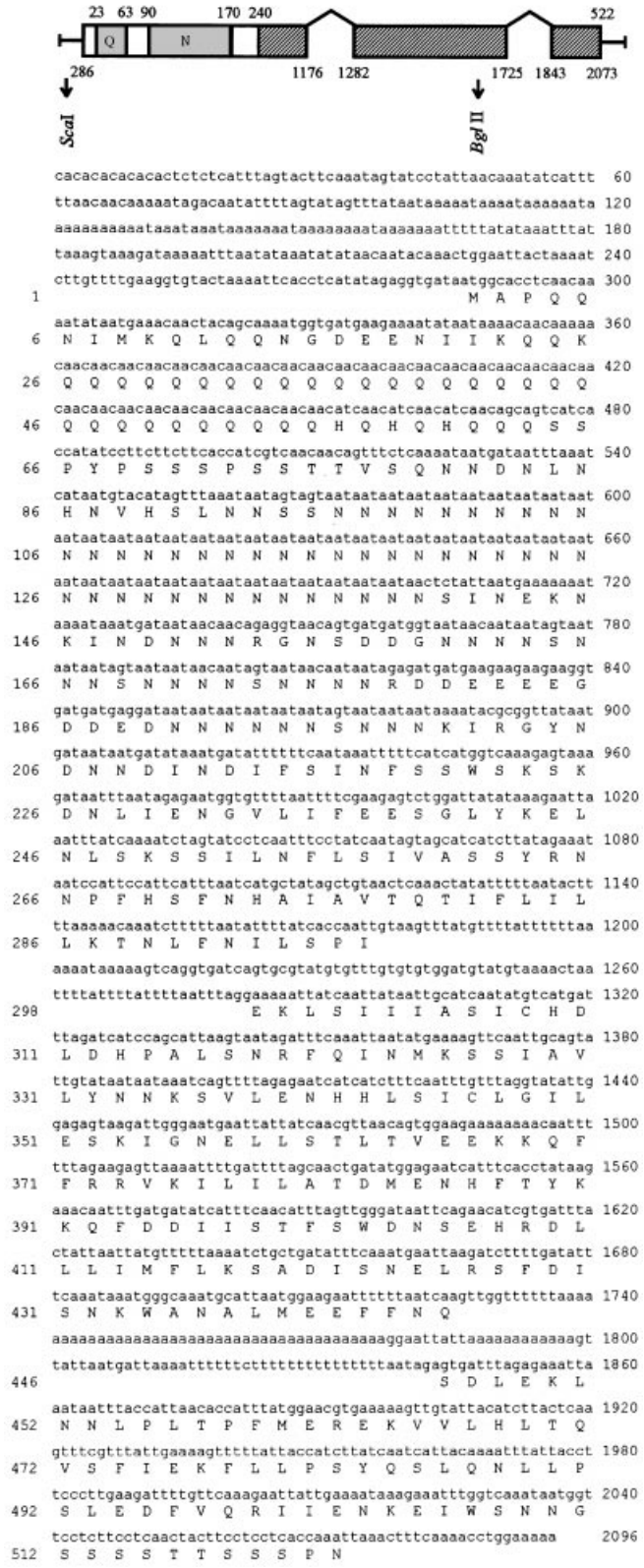


**Figure 1 Scheme and composite sequence of DdPDE3**

Top panel: scheme of DdPDE3. The coding region is shown as a box; the 5′and 3′ untranslated regions are depicted as lines. The putative catalytic domain is shown hatched and the stretches of polyglutamine and polyasparagine are indicated as Q and N respectively. The upper numbers refer to amino acids; the lower numbers indicate nucleotides. Southern blot analysis with the indicated restriction sites *Sca*I and *Bgl*II revealed the predicted 1.7 kb fragment. Lower panel: complete nucleotide and deduced amino acid sequence. The basis of the sequence, including the 5′ and 3′ ends, was cDNA clone 116-6, which was verified and extended by sequences from the *Dictyostelium* database by using three cDNA clones and nine genomic clones that collectively spanned the complete sequence, revealing two introns in the putative catalytic domain. The protein sequence is shown in single-letter code below the nucleotide sequence. Nucleotide and protein numberings are presented at the right and left respectively. Numbering of the protein starts at the first methionine; a second in-frame methionine residue is found at position 8.

starved for 1 h in 10 mM phosphate buffer, pH 6.5, at a density of $10^7$ cells/ml. Cells were washed and resuspended in ice-cold lysis buffer [20 mM Hepes/NaOH/1 mM EGTA (pH 7.0)] to a density of $10^8$ cells/ml, then lysed by forced filtration through a Nucleopore filter (pore size 0.45 $\mu$m). The lysate was centrifuged for 5 min at 14000 ***g***; the supernatant was used in the PDE assays.

### Assays for PDE, cGMP response and chemotaxis

PDE activity was assayed in mixtures of 100 $\mu$l containing PDE buffer [20 mM Hepes/NaOH/1 mM $MgCl_2$ (pH 7.0)], 10 nM [$^3$H]cAMP or [$^3$H]cGMP, unlabelled cAMP or cGMP as indicated, and 30 $\mu$l of enzyme (diluted lysates from *E. coli* or *Dictyostelium* to achieve between 10 and 30 % hydrolysis). The reactions were conducted at 22 °C for 15 min, terminated by boiling for 1 min, and followed by dephosphorylation of the hydrolytic product with phosphatase (1 h incubation at 37 °C with 100 $\mu$l of CIP buffer containing 1 unit of CIP). Finally, 500 $\mu$l of a 50 % (w/v) slurry of DOWEX AG1 × 2 was added, samples were incubated for 15 min at 22 °C, centrifuged for 2 min at 14000 ***g***, and the radioactivity in 250 $\mu$l of the supernatant was determined.

For measurement of the cGMP response, cells were starved for 5 h, washed and then resuspended in 10 mM phosphate buffer, pH 6.5, to a density of $10^8$ cells/ml. Cells were stimulated with 0.1 $\mu$M cAMP; at the times indicated, samples of the cell suspension were added to an equal volume of 3.5 % (w/v) $HClO_4$. The cGMP content was measured with a radioimmunoassay in these lysates after neutralization with $KHCO_3$ [23]. Chemotaxis towards cAMP and folic acid was measured by the small-population assay [24].

## RESULTS

### Cloning of DdPDE3

Two stretches of amino acids that are conserved in the putative catalytic domain of mammalian PDEs were used to search for similar sequences in the *Dictyostelium* cDNA database, HDyDHpGTtNqFlVntKSeLAlLYndESVMEnHH and DLSnpTKplplyRrwAELImeEFFxQGDkEKeMG. In these peptide sequences, capital letters show residues that were kept constant, while at the positions of lower case letters conservative substitutions or other amino acids found frequently in PDEs were accepted. With this method we identified a 0.7 kb cDNA, SSB116, that had been partly sequenced. Its deduced amino acid sequence showed a moderate degree of sequence identity with a small part of mammalian PDE enzymes. We determined the complete nucleotide sequence of this 0.7 kb cDNA, which seemed to encode a complete putative PDE catalytic domain. Because the cDNA hybridizes to an mRNA of approx. 1.9 kb (see below), the missing 1.2 kb sequence might encode regulatory domains. Therefore a complete cDNA (clone 116-6) was isolated from a $\lambda$ZAP cDNA library.

The $\lambda$ZAP cDNA 116-6 is 1882 bp in length. At its 3′ end the

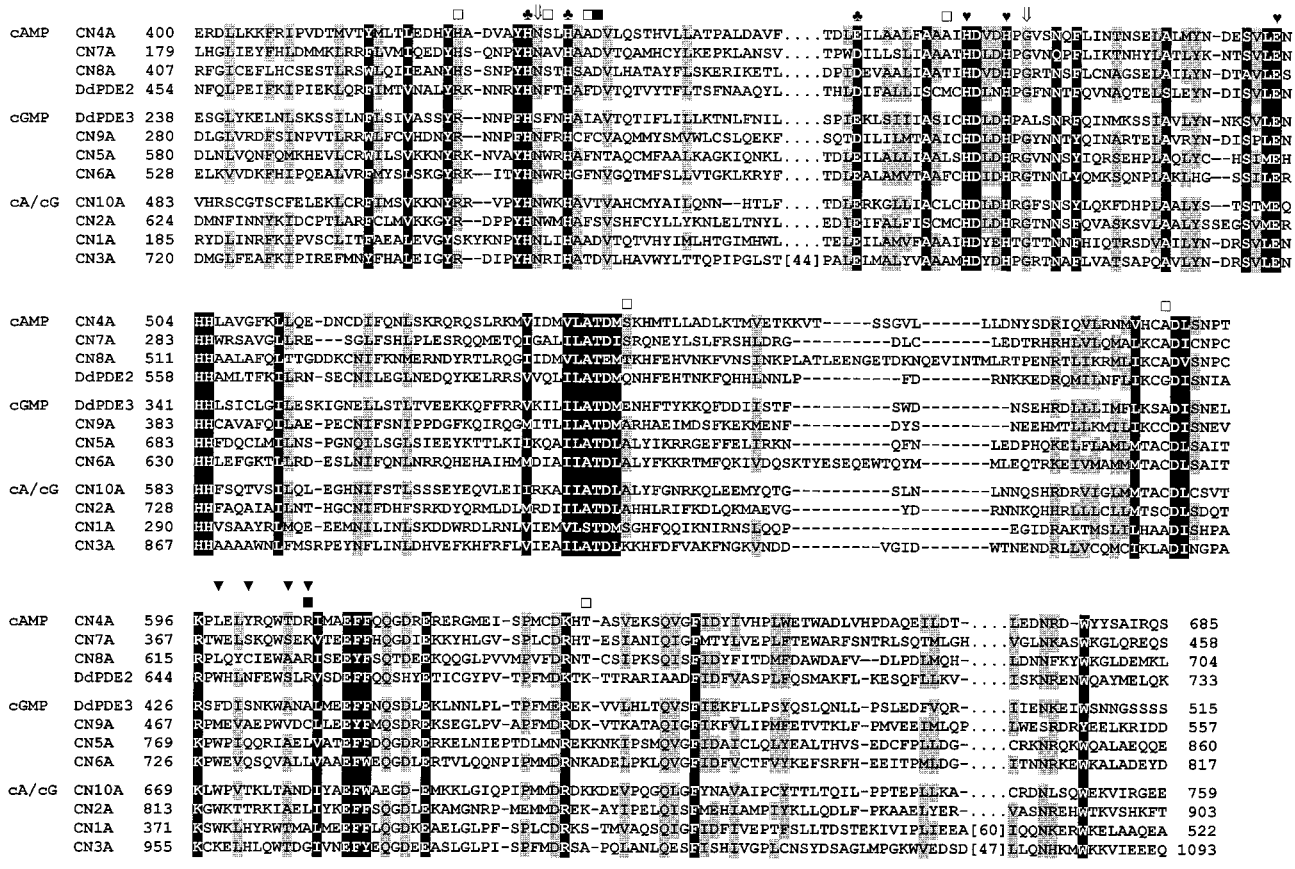


**Figure 2 Alignment of the catalytic domain of DdPDE3 with DdPDE2 (RegA) and ten mammalian PDEs**

All sequences are from published reports (GenBank® accession nos U60170, U40370, U67733, M91667, U68532, AF043731, M27541, L12052, AF048837 and AB020593). Alignment was made with the program ClustalW version 1.8, followed by improvements by hand. Positions where all 12 sequences are identical or conserved are depicted within a black box; positions with 9–11 identical or conserved amino acids are indicated by grey shading. Conserved amino acids are ILVM; STA; DE; NQ; YFW; KRH (single-letter codes). To improve the alignment, insertions of 44 and 47 residues in PDE3A and 60 amino acids in PDE1 relative to other PDEs have been deleted and are indicated by '[44]', '[47]' and '[60]' respectively. The symbol ⇓ indicates amino acids that are different in DdPDE3 relative to all other PDEs; ♣ and ♥ indicate two metal-binding sites. Open squares indicate amino acids that are conserved within the group of mammalian cAMP-specific or cGMP-specific enzymes, but are different between the groups; the filled squares indicate two amino acids that are conserved in all cAMP-specific enzymes, including DdPDE2. The ▼ symbols indicate amino acids that have been mutated in PDE5 [43] to investigate the specificity of substrate binding. Numbers indicate the amino acid positions in the complete proteins.

sequence is nearly identical with EST SSB116 but 50–200 bp shorter than other EST clones identified subsequently. During this project, genomic sequences became available in the *Dictyostelium* genome-sequencing project that collectively spanned the complete cDNA 116-6. Comparison of the genomic sequences and the cDNA reveals the presence of two introns within the catalytic domain (Figure 1). Conceptual translation of cDNA 116-6 reveals an open reading frame (ORF) of 522 amino acid residues, predicting a protein of 60 kDa. The first in-frame methionine residue is located at bp 286 and is preceded by stop codons in all reading frames (Figure 1). A second in-frame methionine is found at position 8. The nucleotide sequences around these ATG codons both comply with the *Dictyostelium* consensus sequence observed at the start codon for translation. The N-terminal 200-residue part of the protein is very rich in asparagine and glutamine residues, with two stretches of about 20 glutamine and 50 asparagine residues respectively, which are interrupted sparsely by other amino acids. Many *Dictyostelium* proteins contain polyglutamine or polyasparagine sequences of unknown function. The putative catalytic domain of about 300 residues follows the N-terminal part of the protein. No other sequence elements are discernible in this protein.

We were concerned about possible cloning artifacts, which we have observed previously owing to the presence of polyasparagine or polyglutamine repeats that are encoded by long stretches of AAT and CAA respectively. It is conceivable that during cDNA synthesis the fusion of two cDNA species occurred at such stretches. This concern was fuelled by a 0.7 kb EST cDNA, SSL142, that was identical at its 5′ end with bp 71–594 of the full-length cDNA 116-6 but showed no identity with DdPDE3 at its 3′ end. Furthermore, we observed a deletion of eight AAT codons in one of the genomic sequences. Unfortunately, none of the genomic clones spanned the repeated sequences. The λZAP cDNA 116-6 predicted restriction sites for *Sca*I and *Bgl*II at bp 24 and 1665 of the putative genomic sequence (see Figure 1). Genomic DNA was digested with these enzymes. A Southern blot revealed a band of the predicted size (results not shown). Together with the observation that the cDNA and mRNA have a comparable size, this suggests that the isolated full-length cDNA was genuine and implies that the deduced protein did not harbour clearly identifiable protein motifs other than the putative catalytic domain. A composite nucleotide sequence assembled from the currently known cDNA and genomic sequences is presented in Figure 1.

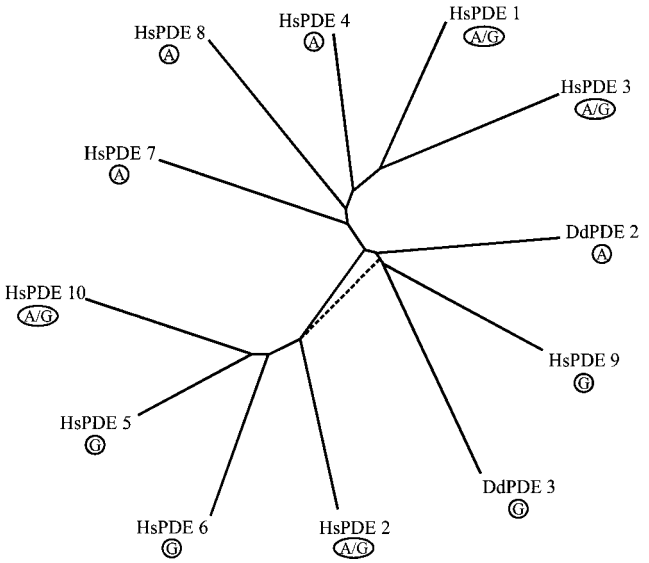


**Figure 3 Family tree of PDE**

With the use of the aligned sequences as presented in Figure 2 (not including the tree inserts), a distance matrix was calculated by using ProtDist with Kimura's approximation. Phylogenies were estimated with the obtained distance matrix by using the Fitch program from the PHYLIP package, version 3.5 [47]. The same results were obtained when the Dayhoff PAM matrix was used instead of Kimura's approximation to calculate the distance matrix, except for the location of DdPDE2 relative to HsPDE7 and HsPDE2 (indicated by the broken line). A and G denote that the enzyme hydrolyses cAMP and cGMP respectively.

### Catalytic domain

Figure 2 shows an alignment of the putative catalytic domain of DdPDE3 with *Dictyostelium* RegA (DdPDE2 [11,12]) and mammalian PDE enzymes [25–36]. The sequence identities found within PDE families are also found in DdPDE3. For instance, of 40 residues that are identical or have conserved substitutions in all other PDE enzymes, 38 are also present in DdPDE3. The exceptions are the substitution of serine for a conserved asparagine residue at position 270, and of alanine for a conserved glycine residue at position 315 of DdPDE3 ( ⇓ in Figure 2). Two motifs of bivalent-cation-binding sites ($HX_3HX_{24–26}E/D$) conserved in PDEs are also observed in DdPDE3 [37].

A BLAST search with the putative catalytic domain of DdPDE3 yields significant scores with several subtypes of PDE from many organisms. At present, 10 PDE families have been identified in mammals (PDE1–10); each family might display several genes (types A, B, and so on) of which different splice variants are known. The catalytic domains of PDE families show an amino acid sequence identity of approx. 45 %, whereas the identity between members of one family is generally approx. 90 %. To limit the discussion we compare DdPDE3 with human type A PDE sequences only. DdPDE3 shows the highest similarity with HsPDE9 (BLAST score 195), followed by *Dictyostelium* DdPDE2 (score 169). The lowest identity (approx. 110) is observed with HsPDE3 and HsPDE6, whereas the other PDEs show an intermediate identity (BLAST score 130–150). By using the alignment of the catalytic domains as presented in Figure 2, we analysed the data with the Kumari method in ProtDis to build a family tree composed of *Dictyostelium* and human PDE sequences. Figure 3 reveals three subgroups of PDE enzymes. One subgroup contains the cAMP-specific enzymes HsPDE4, HsPDE7 and HsPDE8 and the dual-specificity enzymes HsPDE1 and HsPDE3. A second subgroup contains the cGMP-specific enzymes HsPDE5 and HsPDE6 and the dual-specificity enzymes HsPDE2 and HsPDE10. In the third group DdPDE3 is placed together with the cAMP-specific DdPDE2 and the cGMP-specific HsPDE9. The position and composition of the first two groups are well defined by different methods (see the legend to Figure 3), but the position of the third group relative to the other two groups is less well defined; the Dayhoff method places DdPDE2 somewhat closer to HsPDE7 than does Kimura's method. The analysis indicates that there is no strict separation between cAMP-specific, cGMP-specific and dual-specificity enzymes at the amino acid level.

### Expression of DdPDE3 catalytic domain in *E. coli*

The N-terminal 200 residues of DdPDE3 are composed nearly exclusively of repeated Asn/Gln sequences that might cause difficulties in expressing the protein in *E. coli*. Expression was therefore started at residue 202, approx. 36 amino acids before the putative catalytic domain of the enzyme. Lysates from vector-control and DdPDE3-transformed *E. coli* cells were assayed for PDE activity with [$^3$H]cAMP or [$^3$H]cGMP as a substrate. DdPDE3 expression resulted in a moderate increase in cAMP-hydrolytic activity (means $\pm$ S.D., $n = 3$) from $0.94 \pm 0.06$ to $3.0 \pm 0.4$ pmol/min per mg of protein (Figure 4A). In contrast, the hydrolysis of cGMP increased from a barely detectable $0.024 \pm 0.04$ pmol/min per mg in control lysates to as much as $50 \pm 4$ pmol/min per mg in lysates containing recombinant DdPDE3. Thus the catalytic domain of DdPDE3 hydrolyses cGMP approx. 25-fold faster than it hydrolyses cAMP, both at 10 nM substrate concentration. The specificity of DdPDE3 was investigated further with competition experiments, showing that increasing concentrations of unlabelled cGMP or cAMP inhibit the hydrolysis of [$^3$H]cGMP or [$^3$H]cAMP (Figure 4B). Half-maximal inhibition of the hydrolysis of 10 nM [$^3$H]cGMP was observed at 0.22 $\mu$M cGMP or 156 $\mu$M cAMP. Inhibition of the hydrolysis of the less preferred substrate [$^3$H]cAMP yielded similar values, with half-maximal inhibition at 145 $\mu$M cAMP or 0.33 $\mu$M cGMP. Thus the catalytic domain of DdPDE3 binds cGMP with approx. 650-fold higher affinity than cAMP. An Eadie–Hofstee plot for the data on cGMP hydrolysis yielded a linear curve ($r^2 = 0.99$), indicating that the enzyme showed normal Michaelis–Menten kinetics with a $K_m$ of 0.22 $\mu$M (Figure 4C).

### Expression of DdPDE3 catalytic domain in *Dictyostelium*

The catalytic part of the enzyme was also expressed in *Dictyostelium* by using the extrachromosomal plasmid HK12N. Lysates were assayed in the presence of 5 mM dithiothreitol, which inhibits psdA (DdPDE1) but not the DdPDE3 expressed in *E. coli*. We also included 50 $\mu$M cAMP in the assay; at this concentration the activity of DdPDE3 with [$^3$H]cGMP as a substrate was not inhibited (see Figure 4B), whereas the non-specific DdPDE1 and the cAMP-specific DdPDE2 were inhibited by more than 95 % [12]. Under these assay conditions, lysates from control cells exhibited significant hydrolysis of [$^3$H]cGMP (0.4 pmol/min per mg of protein). From previous experiments we know that this activity is derived predominantly from a cGMP-stimulated cGMP-specific PDE. Lysates from cells transformed with DdPDE3 hydrolyse [$^3$H]cGMP at a rate of 3.6 pmol/min per mg of protein, indicating a 9-fold increase in cGMP-PDE activity. Thus the truncated C-terminal part of DdPDE3 is also active in *Dictyostelium*.

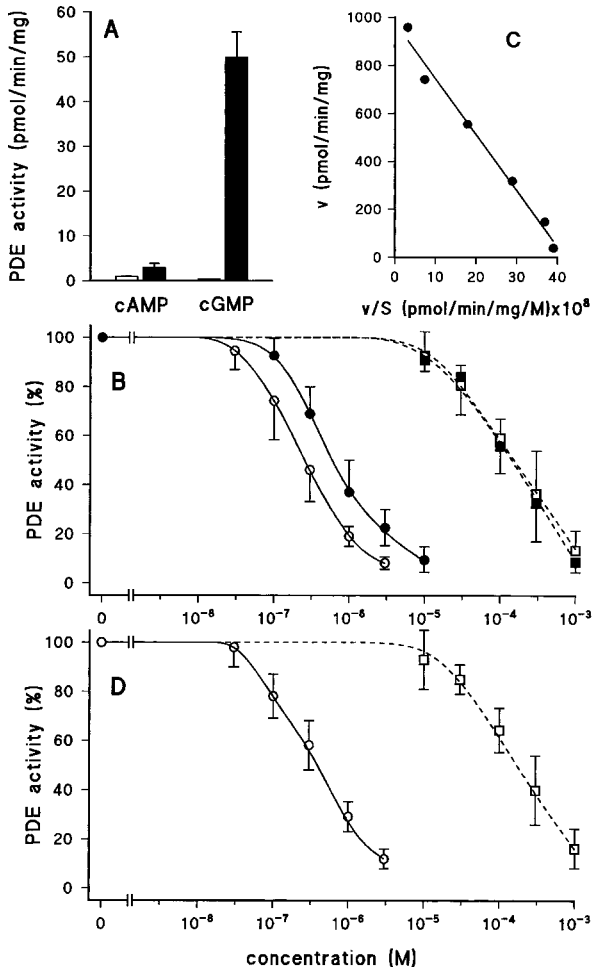


**Figure 4 Substrate specificity of DdPDE3 expressed in *E. coli***

The putative catalytic domain (residues 202–522) was expressed in *E. coli*. Bacteria were lysed and enzyme activity was measured with 10 nM [$^3$H]cAMP or 10 nM [$^3$H]cGMP. (**A**) PDE activity of bacteria containing control vector (open bars) and bacteria expressing DdPDE3 (filled bars). (**B**) Inhibition of PDE activity by cAMP (■,□) and cGMP (●,○) measured in lysates from bacteria expressing DdPDE3; the reactions were performed with 10 nM [$^3$H]cGMP (○,□) or 10 nM [$^3$H]cAMP (●,■). (**C**) Eadie–Hofstee plot of cGMP hydrolysis by DdPDE expressed in *E. coli*. The substrate concentrations used in this experiment were 0.01–3 $\mu$M cGMP. Enzyme concentrations and incubation times were optimized to give no more than 25% substrate conversion. (**D**) Inhibition of [$^3$H]cGMP hydrolysis by DdPDE3 expressed in *Dictyostelium* by cAMP (□) and cGMP (○). Error bars indicate S.D. of three experiments.

Measurements of [$^3$H]cGMP hydrolysis at different concentrations of unlabelled cGMP or cAMP reveal that the catalytic domain of DdPDE3 expressed in *Dictyostelium* has a similar specificity to the catalytic domain of DdPDE3 expressed in *E. coli*. Half-maximal inhibition occurs in *Dictyostelium* at 0.33 $\mu$M cGMP or 207 $\mu$M cAMP, compared with 0.22 $\mu$M cGMP or 156 $\mu$M cAMP for the enzyme expressed in *E. coli* (Figure 4D). Kinetic analysis of the data on cGMP hydrolysis yielded a linear Eadie–Hofstee plot ($r^2 = 0.99$; results not shown), suggesting that the catalytic domain of DdPDE3 expressed in *Dictyostelium* exhibits normal Michaelis–Menten kinetics.

## DdPDE3 mRNA levels in wild-type *Dictyostelium* and mutant *Stm*F

To investigate the expression of DdPDE3, we isolated total mRNA at different times during *Dictyostelium* development and

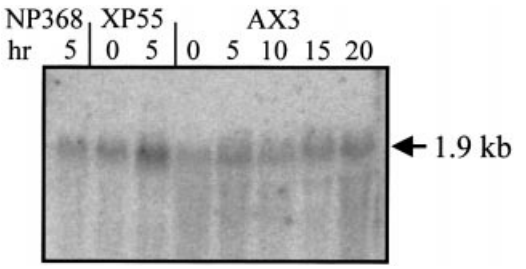


**Figure 5 Northern blot of DdPDE3**

mRNA was isolated from wild-type AX3 cells and mutant *stm*F strain NP368 at the developmental times indicated, then size-fractionated and transferred to a Nytran filter. The blot was probed with the catalytic domain of DdPDE3, detecting an mRNA with an estimated size of approx. 1.9 kb.

**Table 1 cGMP-hydrolysing activity of cells expressing the catalytic domain of DdPDE3**

PDE activity was measured with 10 nM [$^3$H]cGMP in the absence or presence of 1 $\mu$M 8Br-cGMP with lysates from *E. coli* or *Dictyostelium* cells. Results are means ± S.D. for three independent experiments. Abbreviation: n.d., not determined.

| Lysate | PDE activity | | |
|---|---|---|---|
| | Without 8Br-cGMP (pmol/min per mg) | With 8Br-cGMP | |
| | | (pmol/min/mg) | (%) |
| *E. coli* control | 0.24 ± 0.4 | n.d. | n.d. |
| *E. coli* DdPDE3 | 10.4 ± 0.9 | 9.0 ± 0.5 | 87 ± 9 |
| *Dictyostelium* control | 0.41 ± 0.08 | 1.35 ± 0.20 | 332 ± 84 |
| *Dictyostelium* DdPDE3 | 3.59 ± 0.36 | 3.84 ± 0.50 | 107 ± 18 |
| *Dictyostelium stm*F | 0.12 ± 0.02 | 0.13 ± 0.03 | 106 ± 30 |

probed it with a DNA fragment encoding the catalytic domain of DdPDE3 on Northern blots (Figure 5). The results reveal a single band of 1.9 kb present at approximately constant levels throughout development. The size of this band corresponds to the length of the isolated 116-6 cDNA, which is presumed to be full length. The Northern blot also reveals the transcription of the *DdPDE3* gene in *stm*F mutant NP368 and its isogenic parental strain XP55. Mutant *stm*F lacks the cGMP-stimulated cGMP-specific PDE activity, probably owing to a mutation in the structural gene of this enzyme [38]. DdPDE3 seems to be expressed at a normal level in mutant *stm*F. Furthermore, a Southern blot of restriction-enzyme-digested DNA did not reveal a marked difference between the *DdPDE3* gene of wild-type cells and *stm*F mutant cells (results not shown).

Although we cannot exclude point mutations, biochemical results presented below suggest that DdPDE3 is expressed unaltered in mutant *stm*F because the residual cGMP-PDE activity in mutant *stm*F has all the characteristics of DdPDE3. This suggests that DdPDE3 is distinct from the cGMP-stimulated cGMP-PDE that is absent from mutant *stm*F.

## PDE activity in wild-type and *stm*F mutant *Dictyostelium* cells

The hydrolysis of [$^3$H]cGMP in mutant *stm*F was approx. 25% of the activity in the isogenic wild-type strain XP55, measured at 10 nM [$^3$H]cGMP (Table 1). The inhibition of [$^3$H]cGMP hydrolysis by unlabelled cGMP and unlabelled cAMP was used to

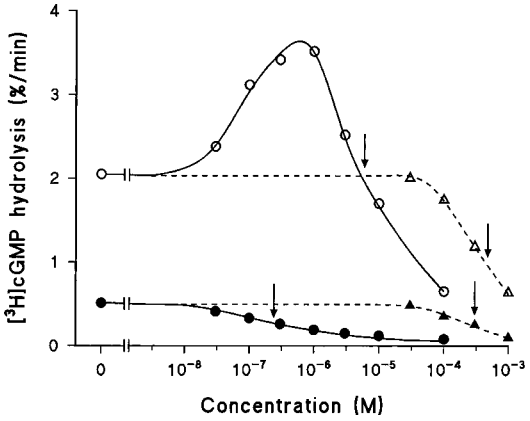


**Figure 6 Hydrolysis of cGMP in wild-type XP55 and mutant *stm*F strains**

PDE activity of XP55 (○,△) or *stm*F (●,▲) was measured with 10 nM [³H]cGMP in the presence of 5 mM dithiothreitol and 50 $\mu$M cAMP; different concentrations of cGMP (●,○) or additional cAMP (▲,△) were included as indicated. Arrows indicate concentrations giving half-maximal inhibition. The results presented in the text on half-maximal inhibition by cAMP take into account the 50 $\mu$M cAMP present in all incubations.

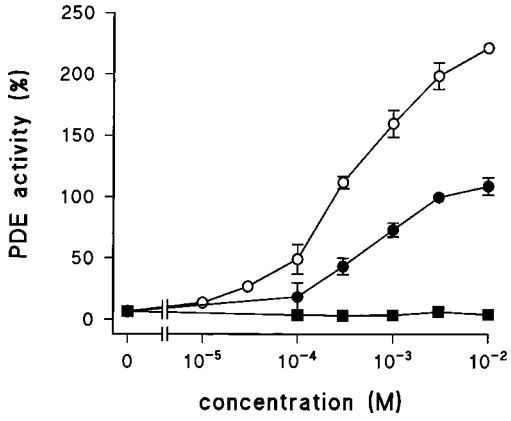


**Figure 7 Activity of DdPDE3 in the presence of bivalent cations**

Enzyme activity of the catalytic domain of DdPDE3 expressed in *E. coli* was measured with 10 nM [³H]cGMP in the presence of the indicated concentrations of $MgCl_2$ (●), $MnCl_2$ (○) or $CaCl_2$ (■); the control without bivalent cations contained 1 mM EDTA. The results are presented relative to the activity with 1 mM $MgCl_2$. Enzyme preparations of *Dictyostelium* wild-type cells, *Dictyostelium* cells overexpressing DdPDE3 and *Dictyostelium* mutant *stm*F showed a similar bivalent cation dependences. Error bars indicate S.D. of three experiments.

characterize the enzyme activities in *stm*F and wild-type XP55 (Figure 6). The addition of low concentrations of unlabelled cGMP to the incubations of wild-type lysates resulted in an increase in the hydrolysis of [³H]cGMP, whereas high cGMP concentrations led to an inhibition of [³H]cGMP hydrolysis. Analysis of these data yields an activation constant, $K_a$, of 0.11 $\mu$M cGMP, a $K_m$ of 5.5 $\mu$M cGMP and a $V_{max}$ of 350 pmol/min per mg of protein for the activated enzyme. These kinetic data are similar to those obtained previously for the partly purified cGMP-stimulated cGMP-PDE from *Dictyostelium* [39]. It was this cGMP-stimulated enzyme activity that was shown to be absent from *stm*F mutant NP368 [14]. Indeed, the residual cGMP-hydrolysing activity of *stm*F was not activated by cGMP; low concentrations of unlabelled cGMP inhibited the hydrolysis of [³H]cGMP (Figure 6). Kinetic analysis reveals Michaelis–Menten kinetics with a $K_m$ of 0.27 $\mu$M cGMP and a $V_{max}$ of 2.2 pmol/min per mg of protein. Inhibition of the hydrolysis of [³H]cGMP by cAMP occurred only at high concentrations, both for the enzyme activity of XP55 and for that of *stm*F cells, yielding half-maximal inhibition at 550 and 370 $\mu$M cAMP respectively.

### Regulation of DdPDE3 by 8Br-cGMP, bivalent cations and IBMX

Previous experiments have shown that the major cGMP-hydrolysing activity in *Dictyostelium* lysates is activated by cGMP with a cyclic-nucleotide specificity that is different from the specificity of hydrolysis [40]. For instance, 8-Br-cGMP is a potent activator of cGMP hydrolysis ($K_a = 0.07$ $\mu$M) but interacts poorly with the catalytic site ($K_i = 50$ $\mu$M). The experiments presented in Table 1 investigate the effect of 8Br-cGMP on [³H]cGMP hydrolysis. In lysates prepared from wild-type cells, 8Br-cGMP stimulates cGMP-PDE activity approx. 3-fold. In contrast, 8Br-cGMP does not alter the cGMP-hydrolysing activity of the DdPDE3 catalytic domain expressed in *E. coli* or *Dictyostelium*. This observation is consistent with the linear Michaelis–Menten kinetics of DdPDE3. 8Br-cGMP also does not affect cGMP hydrolysis by the residual PDE activity of *stm*F.

The amino acid sequence of DdPDE3 exhibits two putative binding sites for bivalent metal ions. DdPDE3 expressed in *E. coli* showed minimal activity in the absence of bivalent cations (Figure 7). Increasing concentrations of $Mg^{2+}$ enhanced the activity, yielding maximal cGMP hydrolysis at 3–10 mM $MgCl_2$. $Mn^{2+}$ was a more potent activator of DdPDE3 than $Mg^{2+}$, whereas $Ca^{2+}$ did not enhance enzyme activity. This cation specificity has been observed in other PDE enzymes, notably in PDE9A [34], and is supported by the proposed two metal-binding sites that have been deduced from the primary structure. The cation specificity of cGMP hydrolysis by *Dictyostelium* lysates is essentially identical with that of the catalytic domain of DdPDE3 expressed in *E. coli*. This was observed for lysates from wild-type cells, from DdPDE-overexpressing cells and from mutant *stm*F cells (results not shown).

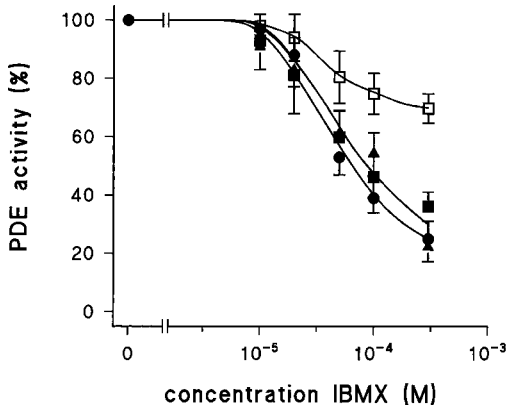


**Figure 8 Effect of IBMX on DdPDE3**

PDE activity was measured with 10 nM [³H]cGMP at different concentrations of IBMX by using lysates of *E. coli* cells expressing the catalytic domain of DdPDE3 (●), *Dictyostelium* wild-type cells (□), *Dictyostelium* cells overexpressing the catalytic domain of DdPDE3 (▲) and *Dictyostelium* mutant *stm*F cells (■). The results are presented relative to the activity without IBMX; see Table 1 for absolute activities. Error bars indicate S.D. of three experiments.

The effect of the PDE inhibitor IBMX on enzyme activity was tested for the different enzyme preparations (Figure 8). IBMX

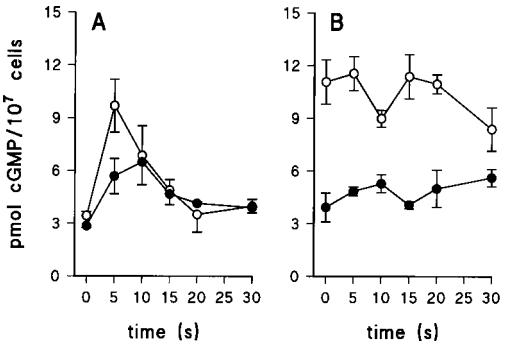


**Figure 9 Effect of DdPDE3 overexpression on cGMP levels *in Dictyostelium* cells**

After starvation for 5 h, cells were stimulated with 0.1 $\mu$M cAMP. At the times indicated, samples were quenched in $HClO_4$ and used to measure cGMP levels. (**A**) Wild-type HK12N cells (○) and DdPDE3-overexpressing cells (●). (**B**) Cells overexpressing *Dictyostelium* guanylate cyclase DdGCA (○) and DdGCA-overexpressing cells that also overexpressed DdPDE3 (●). Error bars indicate S.E.M. of three to five experiments.

inhibited cGMP hydrolysis by the catalytic domain of DdPDE3 expressed in *E. coli*; half maximal inhibition was obtained at approx. 60 $\mu$M IBMX. Slightly higher IBMX concentrations were needed to obtain the same inhibition of cGMP hydrolysis by the catalytic domain of DdPDE3 overexpressed in *Dictyostelium*. In contrast, cGMP hydrolysis in lysates prepared from wild-type cells was poorly inhibited by IBMX, suggesting that the cGMP-stimulated cGMP-PDE is relatively insensitive to IBMX inhibition. Hydrolysis of cGMP by the residual activity of mutant *stm*F seemed to be inhibited by IBMX with the same sensitivity as exhibited by the activity of DdPDE3 overexpressed in *Dictyostelium* (Figure 8).

### Phenotype of DdPDE3-transformed *Dictyostelium* cells

Cells overexpressing the catalytic domain of DdPDE3 showed normal cell aggregation and multicellular development (results not shown). In addition, chemotaxis towards cAMP was not significantly different from that of control cells. To evaluate these observations, we measured basal and cAMP-stimulated cGMP levels. In DdPDE3-overexpressing cells, basal cGMP levels were reduced slightly from $3.44 \pm 0.23$ to $2.87 \pm 0.11$ pmol/$10^7$ cells relative to control-transformed cells; this difference is not statistically significant (Figure 9A). The cAMP-mediated cGMP response is significantly smaller in DdPDE3-overexpressing cells. For a better understanding of the role of cGMP in *Dictyostelium* we also overexpressed a *Dictyostelium* guanylate cyclase (DdGCA) (J. Roelofs, H. Snippe, R. G. Kleineidam and P. J. M. Van Haastert, unpublished work), resulting in an approx. 3-fold elevation of basal cGMP levels (Figure 9B). When the catalytic domain of DdPDE3 was overexpressed in these DdGCA-expressing cells, we observed a pronounced decrease in the cGMP concentration to nearly the basal levels of wild-type cells, clearly demonstrating that DdPDE3 is active *in vivo*. A cAMP-mediated cGMP response was not observed in DdGCA-overexpressing cells, possibly owing to the high constitutive guanylate cyclase activity in these cells. The cAMP-mediated cGMP response remained absent when DdPDE3 was also overexpressed in DdGCA-expressing cells.

## DISCUSSION

Spaciotemporal changes in intracellular cGMP levels might have a crucial role in chemotactic signalling of *Dictyostelium* cells to extracellular cAMP. It is therefore very important to thoroughly characterize guanylate cyclase and cGMP-specific PDE activities at a molecular level. As yet, not all cGMP-PDE activities are accounted for in *Dictyostelium*. Here we characterize a cGMP-specific PDE, DdPDE3, that is not activated by cGMP. This PDE is the third *PDE* gene cloned in *Dictyostelium*, and the first cGMP-specific enzyme cloned in lower eukaryotes.

DdPDE1, encoded by the *psdA* gene [41,42], is a glycoprotein that is secreted or becomes localized on the cell surface. The deduced amino acid sequence of DdPDE1 shows no sequence similarity with other PDEs, suggesting that it evolved independently from all other PDEs. The enzyme is inhibited by dithiothreitol but not by IBMX or other compounds known to inhibit mammalian PDEs. It hydrolyses cAMP and cGMP at approximately the same rate with slight negative co-operativity and an apparent $K_m$ of approx. 1 $\mu$M.

DdPDE2, encoded by the *regA* gene [11,12], was identified in a genetic screen for suppressors of developmental mutants. The deduced amino acid sequence predicts a protein with a response regulatory domain and a PDE catalytic domain that shows strong sequence similarity to that of mammalian PDEs. Enzyme assays with the expressed protein demonstrate that DdPDE2 is a cAMP-specific PDE with a $K_m$ of approx. 5 $\mu$M cAMP. IBMX inhibits DdPDE2 with an $IC_{50}$ of approx. 35 $\mu$M. The enzyme is expressed throughout development, although at lower levels during growth.

### DdPDE3

DdPDE3 was identified through the *Dictyostelium* sequencing projects. Cloning of the full-length cDNA and expression studies of the putative catalytic domain characterize DdPDE3 as a cGMP-specific PDE that is not activated by cGMP. The protein consists merely of a C-terminal catalytic domain of approx. 300 amino acids connected to an N-terminal domain of approx. 200 amino acids predominantly harbouring polyglutamine and polyasparagine stretches. No other protein sequence motifs could be discerned. The catalytic domain of DdPDE3 shows a high affinity for cGMP as a substrate, with a $K_m$ of 0.22 $\mu$M; it is approx. 600-fold more specific for cGMP than for cAMP. The enzyme requires bivalent cations for enzyme activity, with a preference for $Mn^{2+}$ over $Mg^{2+}$, and is inhibited by IBMX with an $IC_{50}$ of approx. 60 $\mu$M.

### Catalytic domain of DdPDE3

The catalytic domains of all PDE enzymes identified so far are positioned at the C-terminus of the protein; regulatory elements, when present, are located at the N-terminus. When comparing the *Dictyostelium* DdPDE2 and DdPDE3 sequences with ten human PDE sequences with the use of computer programs, we noticed that the putative catalytic domains are well aligned up to residue 653 in HsPDE4 (see Figure 2). Inspection by eye clearly identified an additional conserved region that was not previously recognized. This region ends at a conserved Trp and is located just before the C-terminus of many PDE enzymes. In HsPDE1 and HsPDE3 there is an insertion in this region of 60 and 47 residues respectively.

Multiple sequence alignment of the catalytic domains of DdPDE2, DdPDE3 and HsPDE1–10 reveals two distinct groups (HsPDE1, HsPDE3, HsPDE4, HsPDE7 and HsPDE8, and

HsPDE2, HsPDE5, HsPDE6 and HsPDE10), and a third group (HsPDE9 and DdPDE2/3) with less well defined branching (Figure 3). The first group contains the cAMP-specific human enzymes and two dual-specificity enzymes, whereas two cGMP-specific enzymes are placed in the second group together with two dual-specificity enzymes. It is possible that the dual-specificity enzymes HsPDE1 and HsPDE3 evolved from or together with the cAMP-specific enzymes and that HsPDE2 and HsPDE10 evolved from cGMP-specific enzymes.

The notion of the close relationship between PDEs that are either cAMP-specific (DdPDE2) or cGMP-specific (HsPDE9 and DdPDE3) might help in the recognition of the amino acids that provide substrate specificity. The sequence alignment of the mammalian enzymes identifies seven amino acids that are different between cAMP- and cGMP-specific PDEs but are conserved within these groups (indicated by open squares in Figure 2). However, on the inclusion of the *Dictyostelium* DdPDE2 and DdPDE3 sequences in this analysis, none of the seven amino acids remain conserved within the groups. For example, the first potentially selective amino acid (at position 426 of HsPDE4) is histidine in mammalian cAMP-PDEs and arginine in mammalian cGMP-PDEs; however, the cAMP-specific DdPDE2 contains an arginine residue. In contrast, two amino acids are conserved in all cAMP-specific enzymes and differ from the cGMP-PDE or cGMP/cAMP-PDE isoenzymes: Asp and Arg/Lys at positions 439 and 607 of HsPDE4 respectively (filled square in Figure 2). However, in cGMP-specific enzymes no conserved amino acids are present at these two positions. Conversely, there is not a single amino acid that is conserved in all cGMP-PDEs while differing from the amino acids of cAMP-PDE at that position. A detailed mutagenesis study on substrate specificity has been performed with the cGMP-specific PDE5 [43]. Four amino acids were selected based on hydropathy calculations (indicated by ▼ in Figure 2). Conversion of these amino acids into the residues present in the cAMP-specific HsPDE4 (from Trp, Gln, Ala and Leu to Leu, Tyr, Thr and Arg respectively) changed the specificity approx. 100-fold towards cAMP. Interestingly, none of these amino acids are conserved within the group of cAMP-specific or cGMP-specific PDEs. Apparently, substrate specificity in PDEs is not determined by absolutely conserved amino acids. This situation is different from other nucleotide-binding proteins. For instance, in cAMP-dependent and cGMP-dependent protein kinases, specificity is dominated by a conserved Ala and Thr residue respectively [44]. In adenylate and guanylate cyclases, two amino acids determine substrate specificity, Lys and Asp binding ATP, and Glu and Cys binding GTP [45,46].

### Function of DdPDE3 *in vivo*

The cGMP-PDE activity in wild-type *Dictyostelium* lysates is characterized by non-Michaelis–Menten kinetics involving an enzyme that is activated by cGMP. This enzyme has been partly purified and is absent from mutant *stm*F [14]. The genetic (Northern and Southern blots) and biochemical ($K_m$ for cGMP and $IC_{50}$ for IBMX) properties of the catalytic domain of DdPDE3, suggest that DdPDE3 is not identical with the cGMP-stimulated cGMP-PDE. This would imply that the *Dictyostelium* genome still contains at least one other PDE. The *Dictyostelium* sequencing project does not yet provide convincing evidence for the existence of a fourth *PDE* gene. At present the chance of finding a gene with 1 kb of coding sequence (300-residue PDE catalytic domain) is approx. 80–90 %.

The cGMP-PDE activity in mutant *stm*F is approx. 25 % of the cGMP-hydrolysing activity of wild-type cells, both measured at low cGMP concentrations. At higher substrate concentrations the contribution of DdPDE3 to cGMP hydrolysis *in vivo* diminished owing to its high affinity but relatively low $V_{max}$ compared with the cGMP-stimulated PDE. The moderate importance of DdPDE3 in the hydrolysis of cGMP *in vivo* is also demonstrated on overexpression of the enzyme in *Dictyostelium* cells. When the catalytic domain of DdPDE3 is overexpressed approx. 25-fold compared with the endogenous levels of DdPDE3 in wild-type cells, this results in a non-significant decrease in basal cGMP levels and a 33 % decrease in the cAMP-mediated cGMP response. Overexpression of the catalytic domain of DdPDE3 in cells that also overexpress guanylate cyclase DdGCA has a more pronounced effect. The approx. 3-fold increase in cGMP levels owing to DdGCA overexpression is decreased by DdPDE3 to nearly the basal cGMP levels of wild-type cells. These combined results suggest that DdPDE3 is involved mainly in regulating basal cGMP levels and has no predominant role in hydrolysing cGMP that is synthesized on receptor stimulation. This conclusion is supported by the lack of an overt phenotype of the DdPDE3-overexpressing cells.

In summary, we have identified a high-affinity cGMP-specific PDE in *Dictyostelium* that shows similarity to mammalian PDE9, in both its biochemical properties and its amino acid sequence. The enzyme contributes at most 30 % to the hydrolysis of intracellular cGMP; the remainder is contributed by a cGMP-stimulated cGMP-PDE that is absent from mutant *stm*F. Identification and isolation of the gene encoding the latter enzyme will provide the means of full control of cGMP levels in *Dictyostelium* and of establishing the function of cGMP in this organism.

We thank Dr Takahiro Morio and colleagues [supported by the Japan Society for the Promotion of Science (RFTF96L00105) and Ministry of Education, Science, Sports and Culture of Japan (08283107)] for providing us with cDNA EST clones; we also thank the Japanese cDNA consortium (Hokkaido University, University of Tsukuba and Kinki University, Japan) and the genomic DNA consortium (University of Cologne, Cologne, Germany; the Institute of Molecular Biotechnology, Jena, Germany; the Baylor College of Medicine, Houston, TX, U.S.A., and the Sanger Centre, Hinxton, Cambs, U.K.); and Dr Jaco C. Knol for constructive comments on the manuscript.

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Received 30 May 2000/20 September 2000; accepted 27 October 2000